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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*
We have established an *ex vivo* culture system using MCF-10A cells. The doubling time for this cell line in our laboratory is ~3-5 days. We have determined the cytotoxicity of three antibiotics towards MCF-10A cells. The cells were found to be more sensitive to antibiotics than other cell lines we have used experimentally. The effective concentrations for selection of MCF-10A cells are: zeocin, 300µg/ml; puromycin, 0.5 µg/ml; and hygromycin, 40 µg/ml. We have standardized our method for transfection (lipofection) of the MCF-10A cells. Two expression vectors for DNA polymerase beta (polß) have been constructed: pCDNA4-based and pIRES-based. Stable zeocin-resistant MCF-10A clones were isolated following transfection of pCDNA-polß. While endogenous polß protein was detected by Western analyses, no exogenous protein was detectable in these clones. Stable selection using the pIRES-ß/pIRESpuro vector pair was only partially successful, as the MCF-10A, puromycin-resistant clones became senescent. However, Western analyses of polß-pIRES transfected MCF10A cells demonstrated the exogenous expression of His-polß in the clones. These results demonstrate that our proposed experiments are feasible. However, the time frame needed to complete the project may be substantially longer than anticipated, due to the slow growth rate of the MCF-10A cell line and sensitivity to antibiotics.

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Introduction

The goal of this proposal is to develop an *ex vivo/in vivo* experimental system that will allow determination of whether a mutator phenotype is *sufficient* to accelerate neoplastic transformation of an immortalized breast epithelial cell line and/or accelerate malignant progression of a pre-neoplastic cell line. In this approach, overexpression of variant mutator forms of DNA polymerase ß (polß) acts as a surrogate means of generating genetic diversity. Pol ß is the major DNA synthesizing enzyme in base excision repair (BER). DNA lesions resulting from normal cellular metabolism contribute to spontaneous mutations, and are removed by the BER pathway. We hypothesize that intracellular overexpression of polß variant enzymes will increase the level of spontaneous mutagenesis, and result in random mutations of oncogene and tumor suppressor loci in epithelial cells. Our long-term goal is to utilize this system to elucidate the role in breast cancer development of various endogenous conditions which may contribute to genetic instability, such as estrogen metabolism and oxidative stress, both of which form DNA adducts repaired by the BER pathway. This avenue of research is vital to understanding oncogenesis in the majority of sporadic human breast cancers, the etiology of which is not associated with familial genetic defects or gross exposures to environmental chemicals.

Body

Our research accomplishments associated with each task as outlined in the approved Statement of Work are summarized in Table 1. Each entry is explained in detail following the table.

Table 1. Summary of Research Tasks and Accomplishments (Year 1)

Approved Tasks (Year 1)	Individual Experiments	Progress to Date
Task 1: Isolate and characterize polß-overexpressing clones of MCF-10A and MCF-10AT cell lines (months 1-3)	**1A.** Establishment of MCF10A cell culture.	**Complete**
	1B. Transfection of MCF10A cells with expression vectors. 1B1. Construction of polß gene "cassette" 1B2. Construction of polß vectors 1B3. Standardization of transfection conditions	**Complete** Figure 1 Figure 2 Table 2
	1C. Isolation of two stable transfectant clones for each vector. 1C1. Antibiotic cytotoxicity determination. 1C2. Selection of clones	**Partially Complete** Figure 3 In progress
	1D. Measure levels of endogenous and variant polß protein expression in clones by Western analyses of cell extracts	**Complete** Figure 4
Task 2: Quantitate HSV-tk mutation rates in control and polß-overexpressing MCF-10A/AT cell lines (months 3-12)	**2A.** Transfect MCF-10A cell lines with HSV-*tk* shuttle vector DNA. 2A1. Hygromycin toxicity study	**Partially Complete** Figures 5,6
	2B. Purify shuttle vector DNA from MCF10A transfectants	**In progress**

1A. Establishment of MCF10A cell culture.

The first month of this project was devoted to establishing *ex vivo* cell culture practices for the MCF-10A cell line. We obtained cells at passage 91 from the laboratory of Dr. Danny Welch, co-investigator on this project. We now routinely culture this cell line using published media constituents (Soule *et al.*, 1990): DMEM/F12 media supplemented with 5% horse serum, 10 μg/ml insulin, 10 ng/ml EGF, 0.5 μg/ml hydrocortisone, and 100 ng/ml cholera toxin. In our laboratory, the doubling time of the cells is approximately 3-to 5 days. This growth rate is much less than we anticipated when we proposed our task timeline, causing significant delays in our progress.

1B. Transfection of MCF10A cells with expression vectors.

B1. Construction of polß gene "cassette"

Our initial progress toward creating epitope-tagged polß expression vectors was hampered by the incompatibility of restriction enzyme sites among the three cloning vectors pET, pIRES and pCDNA4 (Figure 1A). We therefore adopted a strategy in which new BamHI and BstXI restriction sites could be engineered into the polß insert gene by PCR, thus creating a "pol ß cassette". This cassette then could be moved easily among various vectors, including any we might employ in the future (Figure 1B).

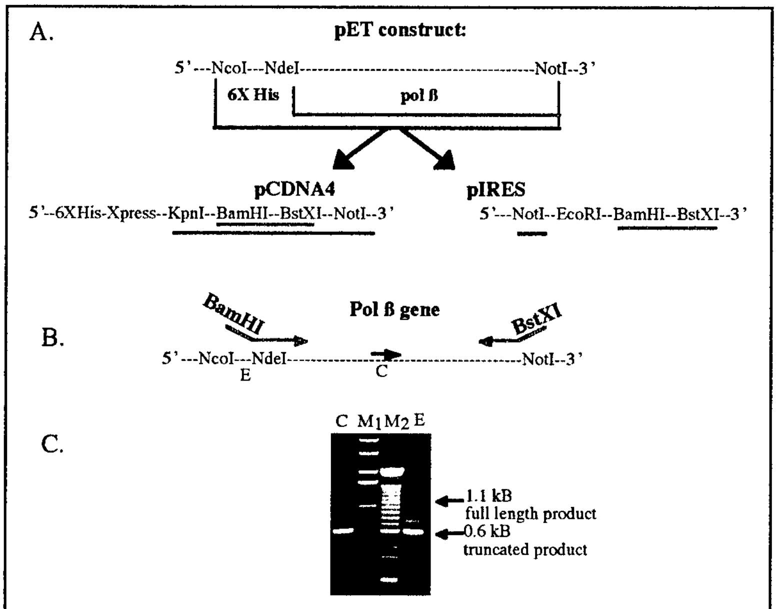

Figure 1. Engineering the polß gene for cloning into mammalian vectors. (A). Schematic of restriction sites in pol ß donor (pET) and recipient (pCDNA4, pIRES) vectors. (B). PCR primers used to introduce new restriction sites (green) into polß gene. (C). PCR products using the BstXI primer and either an internal primer (C) or the experimental BamHI primer (E). M indicates DNA markers.

Unfortunately, we have been unsuccessful in obtaining a full-length polß gene product using this approach. As shown in Figure 1C, the desired 1.1 kb PCR product constitutes a minority of the total PCR product. Alterations in all parameters of the PCR, including polymerase, [$MgCl_2$], annealing temperature, and molar ratio of primers and template DNA, were performed in an attempt to increase the yield of the desired product. We believe that the forward PCR primer (that includes the BamHI site) also primes internally to the gene to yield a truncated product of ~600 bp in size. Thus, future experiments utilizing alternate primers will be needed to engineer the polß gene using this approach.

B2. Construction of polß expression vectors

Human cell expression vectors for polß were created by subcloning an NcoI-NotI fragment from our 6X-histidine tagged, rat polß pET bacterial expression vector into the pIRES puromycin vector (Clontech) (Figures 1A and 2B). This vector was chosen for its bicistronic feature which ensures simultaneous expression of the cloned polß gene and the antibiotic resistance gene. However, we encountered technical difficulties while constructing this vector which delayed its use for the MCF10A cells. Thus, we also cloned an NdeI-NotI fragment of our polß gene from pET into the pCDNA4-HisMax (Invitrogen) expression vector (Figures 1B and 2A). This vector is advantageous in that it encodes two different epitope tags, His and Xpress, for protein detection in human cells.

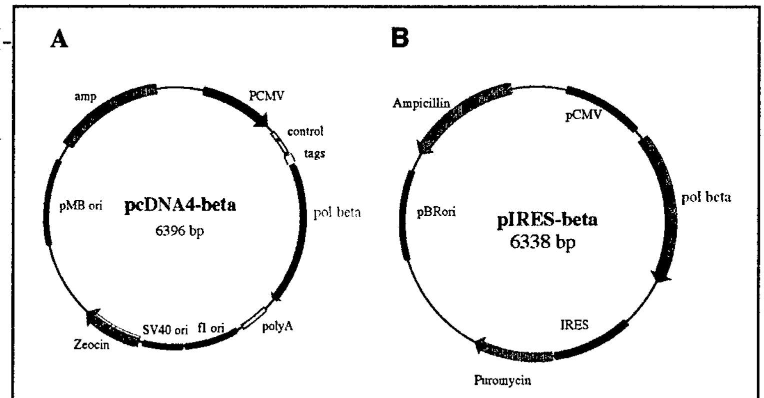

Figure 2. polß expression vectors for MCF-10A cells.

B3. Standardization of transfection conditions.

We have employed the lipofection method to introduce exogenous DNA into MCF10A cells because of the reported higher efficiency and lesser cytotoxicity relative to standard transfection protocols. Two commercially available lipofection reagents were used to transfect MCF10A cells with the control pCDNA4 vector. A transfection efficiency of ~40% was obtained with either reagent (Table 2).

1C. Isolation of two stably transfected clones for each vector and cell line.

C1. Antibiotic cytotoxicity determination.

Our experimental approach requires the isolation of MCF-10A cell clones that have stably integrated our polß expression vectors into the genome. The isolation of such stable transfectants requires the use of an antibiotic resistance marker that is expressed from the polß vector. As a first step towards accomplishing this goal, we performed survival curves of MCF-10A cells in the presence of the appropriate antibiotics: Puromycin for the pIRES based vector and Zeocin for the pCDNA based vector. These experiments were completed over a time frame of 4-5 weeks for each dose curve. Puromycin is extremely toxic to the MCF-10A cell line (Figure 3A); we observed selection with a minimal dose of 0.5 μg/ml puromycin, ten-fold lower than the dose recommended for mammalian cell selection. Zeocin, a bleomycin derivative is less toxic; we observed a minimal selection dose of ~300 μg/ml Zeocin (Figure 3B). In our proposal evaluation, one reviewer stated concern for our use of bleomycin as a selective agent, as this antibiotic is known to cause DNA damage and may elevate our mutation frequencies, thus complicating our data analyses. We fully concur with this viewpoint, but are now faced with a difficult situation, as our choices for antibiotic resistance markers are limited. The pIRES vector is the most desirable system to use for our experiments, but the highly toxic nature of puromycin causes the transfected cells to grow poorly under selective conditions (see below, part C2).

C2. Selection of clones containing human cell expression vectors.

MCF-10A cells were transfected with either the pIRES-ß/pIRESpuro or the pCDNA-ß/pCDNA4 vector pairs using lipofection, and selective pressure applied by the appropriate antibiotic. At this step, we again were unprepared for the extended time frame required to perform this task, relative to our experience with other established cell lines. We attribute this difficulty to the near normal phenotype of the MCF-10A cells and their low passage number; ironically, two of the reasons we chose to do our studies with this cell system. Transfection and stable selection using the pIRES-ß/pIRESpuro vector pair was only partially successful(Table 2). After the initial death of non-transfected cells during growth in puromycin (period of 2-4 weeks), transfected cells were identifiable as small patches of clonal growth. Unfortunately, the presence of puromycin retarded the growth of the cells, and the transfected clones grew very poorly and could not be

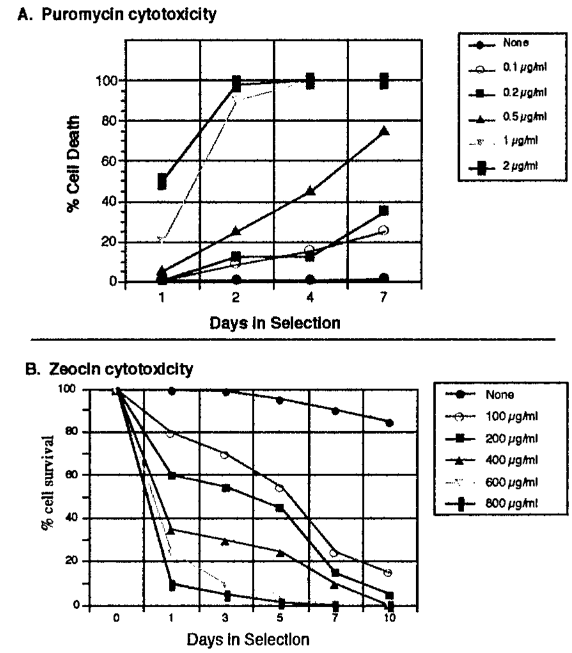

Figure 3. MCF-10A cell survival curves in the presence of selective agents.

Table 2. Summary of Transfection Studies

Experiment	Pol ß Expression Vectors		HSV-tk Shuttle vector
	pIRES	pcDNA4	
Transfection efficiency (lac Z)	No control vector is available	40-45% Fugene 40% Lipofectamine	No control vector is available
Stable transfectants (Antibiotic)	+ (senescent) (Puromycin)	+++ (Zeocin)	++ (Hygromycin)
Western analysis	Positive for exogenous expression of pol ß using anti-His antibody.	Positive for endogenous expression of polß using anti-pol beta antibody; No detection of polß using anti-His antibody.	Not done.
Present status	Efforts to maximize transfection efficiency and cell survival under antibiotic selection.	Cloning polß into pcDNA based vectors with different antibiotic selection.	Optimization of shuttle vector isolation.

expanded. Removal of puromycin did not increase cell growth, and all of these clones underwent senescence. However, we have successfully isolated stable clones of the pCDNA-ß/pCDNA4 vectors over a period of 8-10 weeks (Table 2). These clones could be propagated in the presence of antibiotic (Zeocin) selection and expanded for population analyses.

1D. Measure levels of polß protein expression by Western analyses.

The clones generated in section 1C were analyzed for polß protein expression. Western analyses were performed using anti-polß antibody (NeoMarkers) and cell extracts from the pCDNA-ß clones, pCDNA4 (vector) clones and untransfected MCF10A cells. Endogenous polß was readily detected in all samples (Figure 4A). However, no protein was detected using the same samples and probing with an anti-His antibody (Qiagen) to detect exogenous polß expression from the pCDNA-ß vector (not shown). This may be due to DNA sequence changes in the expression vector or to differences between the His sequence tag and the epitope recognized by the anti-his antibody.

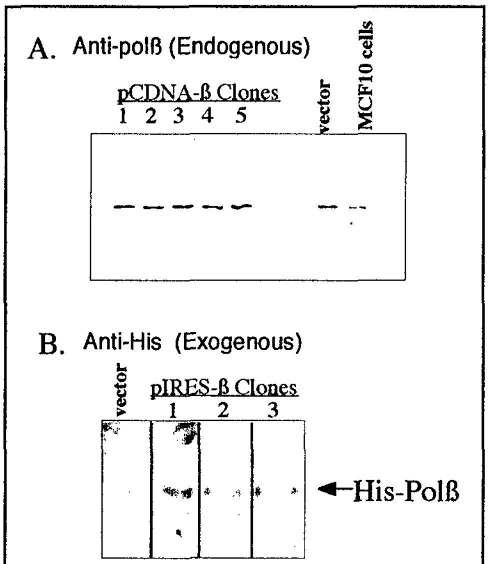

Figure 4. Western analyses of endogenous and exogenous polß expression in MCF-10A cells.

The senescent pIRES-transfected cells were collected in an attempt to monitor exogenous polß protein expression from this vector system. Unfortunately, we were not able to collect enough cells to simultaneously measure endogenous polß levels in these clones. Never-the-less, our western analyses of polß-pIRES transfected MCF10A cells using anti-His antibody (Qiagen) clearly demonstrate the exogenous expression of His-polß in the clones, and the lack of expression in the vector-only transfected clone (Figure 4B). In summary, although we encountered high MCF-10A cell toxicity using puromycin, we can detect exogenous polß expression in the non-proliferative clones. Presently, we are attempting to optimize growth of pIRES-transfected MCF-10 cell clones in puromycin selective medium.

2A. Transfect MCF-10A cell lines with HSV-*tk* shuttle vector DNA.

The oriP-tk shuttle vector pJY102 was constructed for use in the MCF-10A epithelial cell line (Figure 5) under a previous "start-up" grant period. This vector contains the oriP and EBNA-1 sequences from Epstein-Barr virus for episomal replication in epithelial cells; the hygromycin resistance gene for selection of vector-containing human cells; and the thymidine kinase gene from Herpes simplex virus type 1 for mutational analyses.

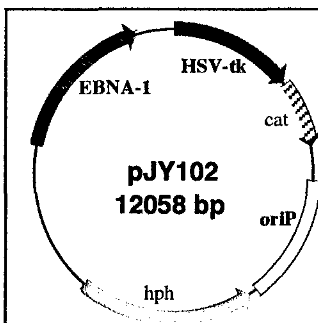

Figure 5. Shuttle vector for mutational analyses.

A1. Hygromycin toxicity study

An initial hygromycin toxicity profile has been generated for our MCF-10A cell line (Figure 6). From these results, we expect that a dose of 40 μg/ml of hygromycin should be sufficient for selection of stable transfectants. The pJY102 vector has been used to transfect MCF-10A cells using the lipofection method, hygromycin-resistant clones have been isolated (Table 2). Experiments are in progress to isolate the shuttle vector from the MCF-10A cells for mutational analyses.

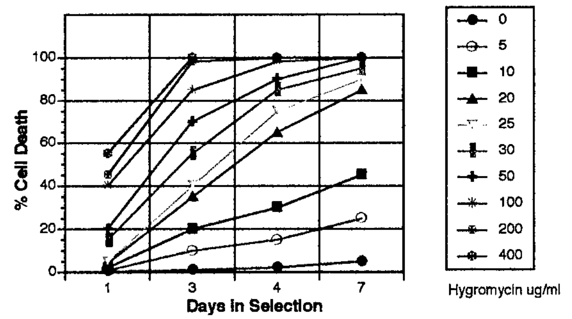

Figure 6. Hygromycin toxicity of MCF-10A cells.

Key Research Accomplishments

- **Establishment of MCF-10A cell culture**
- **Selection and growth of pCDNA-ß/MCF-10A transfectants**
- **Demonstration of exogenous His-polß protein expression in pIRES/MCF-10A transfected clones**

Reportable Outcomes

There were no reportable outcomes of this research during this reporting period.

Conclusions

We submitted this IDEA grant with a hypothesis and an experimental approach to test that hypothesis, but without preliminary data. The results presented in this report demonstrate that our experimental approach is technically feasible. Experiments are underway to optimize all of our conditions, and we are optimistic that we will derive the desired MCF-10 cell line mutational data during the next year. We have recently begun culturing MCF-10AT cells, a derivative of the MCF-10A cell line that has been transformed by expression of the ras oncogene. Experiments utilizing this cell line with the polß expression vectors are in progress.

References

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